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Incidence and features of thrombosis in children with inherited antithrombin deficiency

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ABSTRACT

Pediatric thromboembolism (≤18 years) is very rare (0.07-0.14/10,000/year) but may be more prevalent in children with severe thrombophilia (protein C, protein S or antithrombin deficiency). The aim of this study was to define the prevalence and clinical characteristics of pediatric thrombosis in subjects with inherited antithrombin deficiency. Our observational retrospective multicentric study from two countries recruited 968 patients of any age from 441 unrelated families with genetically, biochemically and functionally characterized antithrombin deficiency. Seventy-three subjects (7.5%) developed thrombosis before 19 years of age. Two high-risk periods for thrombosis were identified: adolescence (12-18 years, n=49) with thrombus localization (lower limb deep venous thrombosis or pulmonary embolism) and triggering factors common to adults (oral contraceptives, surgery or pregnancy); and the neonatal period (<30 days, n=15) with idiopathic thrombosis at unusual sites. The clinical evaluation of pediatric thrombosis in subjects with antithrombin deficiency revealed: i) a high prevalence of cerebral sinovenous thrombosis (n=13, 17.8%), mainly at young age (8 neonates and 4 children <6 years); ii) severe outcome with fatality in six cases (3 neonates, two of them homozygous for p.Leu131Phe). The majority of subjects (76.7%) carried quantitative type I deficiency. This retrospective analysis includes the largest cohort of subjects with inherited antithrombin deficiency so far and provides strong evidence for an increased risk of pediatric thrombosis associated with this thrombophilia (300-fold compared with the general population: 0.41%/year *vs.* 0.0014%/year, respectively). Our results support testing for antithrombin deficiency in children of affected families, particularly in case of type I deficiency.

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Introduction

Thromboembolism is a life-threatening event that significantly contributes to the global disease burden.[1] Age is the main risk factor for developing venous thromboembolism (VTE).[2] Thrombosis in the pediatric population is rare, with incidences ranging from 0.07 to 0.14 per 10,000 children aged ≤18 years per year. Nowadays, these numbers are growing as a result of better diagnosis, improved survival of children with severe underlying diseases, and increased use of invasive procedures and instruments.[3] Pediatric thrombosis is recognized as an important complication of severe medical conditions such as sepsis, cancer, congenital heart disease, and the use of pharmaceutical drugs such as asparaginase and estrogen-containing contraceptives. Surgery and invasive procedures, particularly placement of central venous catheters, are thrombogenic conditions involving around 50% of pediatric VTE, a number that rises to more than 90% in neonates.[4] According to data obtained from case series, case-control studies, registries and cohort studies, thrombophilia is a

known risk factor for pediatric thrombosis. A meta-analysis of these studies and a recent nation-wide survey showed that children with first-onset VTE were more likely to have severe inherited thrombophilia, like deficiencies of natural anticoagulants (antithrombin, protein C and protein S), than controls.[5,6]

Antithrombin deficiency, an autosomal dominant disorder, was the first thrombophilia to be described 50 years ago and so far is associated with the highest risk of thrombosis.[7] The key hemostatic role of this anticoagulant serine protease inhibitor (serpin) explains why heterozygous mutations in *SERPINC1*, the gene encoding for antithrombin, significantly increase the risk of VTE (OR: 20-40)[8] and why the complete absence of antithrombin causes embryonic lethality in mice.[9] However, there is a significant clinical variability among patients with antithrombin deficiency. Patients with quantitative type I deficiency, where the genetic defect disturbs the production or secretion of the variant protein, have a higher incidence of thrombosis compared to patients with qualitative type II deficiency, where the genetic defect allows the production of a variant antithrombin with impaired anticoagulant activity.[10,11] Three different subgroups of type II deficiency can be distinguished: Reactive Site (RS), when the binding of the substrate to the reactive site is affected; Heparin Binding Site (HBS) when the heparin binding domain is altered; and Pleiotropic Effect (PE), with both effects on the protein.[10] Homozygotes have only been described for type II deficiency.[8] Age is an additional risk factor for patients with antithrombin deficiency, as up to 60% of patients develop a thrombotic event before the age of 65.[12] In contrast to adults, less data are available for young subjects as pediatric studies on antithrombin deficiency are mainly restricted to case reports or small patient cohorts, due to the rarity of the disorder.[13-16]

The objective of this study was to investigate the prevalence and clinical characteristics of pediatric thrombosis in a large cohort of subjects with inherited antithrombin deficiency recruited in two countries.

Methods

Ethics

This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Hospital Universitario Reina Sofia (8/2013). Written informed consent was provided.

Patients

During a period of 21 years in Spain (from 1996 to 2017) and 27 years in Belgium (from January 1990 to December 2017), two reference centers for antithrombin deficiency recruited 441 index patients. Initial diagnosis could have been made in another center but was always confirmed by measurements of antithrombin activity (anti-FXa activity <80%) and genetic analysis. In 206 of the index patients, family studies were performed and 527 first and second degree affected relatives were identified and enrolled in the study, generating a final cohort of 968 patients with antithrombin deficiency.

The patient's history was evaluated to record for thrombotic events and possible provoking risk factors such as oral contraceptives, pregnancy, complicated delivery, obesity, immobilization, infection, surgery, and trauma. Information about antithrombotic therapy and family history of thromboembolism was also collected. Results from additional thrombophilic parameters (protein C activity, free protein S antigen, resistance to activated protein C, Factor V Leiden and prothrombin G20210A mutation) were collected when available. Thrombotic events were objectively diagnosed by experienced radiologists through established imaging procedures such as Doppler-ultrasonography, computed or magnetic resonance tomography for venous thrombosis and spiral computed pulmonary angiography or lung perfusion scintigraphy for pulmonary embolism.

Definitions

Pediatric thrombosis was defined as any objectively diagnosed thrombotic event during childhood (≤ 18 years). Pediatric patients were divided into age groups according to the proposed World Health Organization (WHO) classification: neonates from birth to 30 days, infants from one month up to 2 years, children from 2 up to 12 years, and adolescents from 12 to 18 years.[17]

Genetic analysis

Genetic analysis was performed in every patient with reduced antithrombin anti-FXa activity. Genetic variants in *SERPINC1* were identified by sequencing the 7 exons and flanking regions. Gross rearrangements were assessed by multiplex ligation-dependent probe amplification using the *SALSA MLPA Kit P227 SerpinC1* (MRC-Holland). Mutations were described following the Human Genome Variation Society Guidelines (*http://varnomen.hgvs.org/recommendations/*). The GenBank NM_000488.3 cDNA sequence was used as reference sequence. Where available, HGMD accession numbers were mentioned.

Biochemical and functional characterization

Antithrombin anti-FXa activity was determined in citrated plasma by chromogenic methods following the manufacturer's instructions (HemosIL Antithrombin, Werfen, Barcelona, Spain and Innovance Antithrombin, Siemens, Marburg, Germany). Antigen levels were measured by rocket immunoelectrophoresis and/or ELISA.

Analysis of plasma antithrombin forms included crossed immunoelectrophoresis and polyacrylamide gel electrophoresis.

The reported results were performed in samples collected long after the acute event and in absence of any anticoagulant treatment. For the neonatal patients, reported results were performed after the first six months of life, except in one patient who died as a consequence of the thrombotic event (*Online Supplementary Table S1*).

Statistical analysis

Continuous variables were expressed as means and standard deviations and categorical data as counts and percentages. Relative risks and 95% confidence intervals (CI) were calculated using previously published formulas.[18] The significance of differences in continuous variables was tested by Mann-Withney test. Kaplan-Meier survival curves were used to illustrate the difference in thrombosis-free survival among different groups. *P*<0.05 was considered statistically significant. Statistical and graphical analysis were performed with GraphPad Prism version 7.03 (GraphPad Software, San Diego CA, USA) and SPSS, version 21 (Chicago, IL, USA).

Results

Seventy-three patients (37 from Spain and 36 from Belgium) out of 968 subjects with congenital antithrombin deficiency developed a first thrombotic event before the age of 19 (Table 1 and Figure 1) corresponding to a fre-

quency of 7.5% or 4.32 cases/1000-patient years. As thrombotic events in children are unusual, further investigations are nearly always performed. As a result, 54.8% of pediatric patients included in our study were the probands of the affected families (40 of 73). At first thrombotic event, 15 of the patients were neonates, one was an infant, eight were children and 49 were adolescents (Figure 2). Almost half of these events were provoked by additional risk factors (35 of 73, 47.9%) and mainly in adolescents (25 of 35, 71.4%) (Table 1). A detailed description of all 73 cases is shown in *Online Supplementary Table S1*.

Analysis by sex showed a slightly higher incidence of thrombotic events in males than in females (54.8% *vs.* 45.2%, respectively) (Table 1). This difference was even more pronounced when considering thrombosis at early age: 10 out of 15 neonates with thrombosis (66.7%) were male (*Online Supplementary Table S1*). When restricting the analysis to children under the age of 11 years, thus excluding the role of estrogen-associated thrombosis, males showed a significantly higher risk for the development of pediatric thrombosis than females (OR 3.2; 95%CI: 1.3-78; P=0.012). These differences in thrombo-

Table 1. Characteristics of the patients with antithrombin deficiency and pediatric thrombosis.

Pediatric thrombosis	SPAIN	BELGIUM	TOTAL	Provoked	Antithrombin (anti-FXa activity)	Unusual thrombosis	Deaths
Cases	37 (6.1%)	36 (10.1%)	73 (7.5%)	35 (47.9%)	52.3±10.8%	17 (23.3%)	6 (8.2%)
Age at first thrombotic event (years)	11.4 ± 7	11.5 ± 7	11.4 ± 7	12.4 ± 7	–	2.6 ± 5.3	5.6 ± 8
Females	13 (35.1%)	20 (55.5%)	33 (45.2%)	21 (63.6%)	53.0±12.5%	6 (18.8%)	3 (9.1%)
Males	24 (64.9%)	16 (44.5%)	40 (54.8%)	14 (35%)	51.7 ±9.2%	11 (27.5%)	3 (7.5%)
Thrombosis in adolescence (12-18 years)	25 (66.7%)	24 (66.7%)	49 (67.1%)	25 (51%)	52.3±8.5%	1 (2%)	1 (2.0%)
Thrombosis in neonates (< 30 days)	6 (16.2%)	9 (25%)	15 (20.5%)	8 (53.3%)	46.2±13.4%	11 (73.3%)	3 (20%)
CSVT	7 (18.9%)	6 (16.7%)	13 (17.8%)	7 (53.8%)	51.5±9.2%	13 (100%)	1 (16.6%)
Deaths	5 (13.5%)	1 (2.8%)	6 (8.2%)	2 (33.3%)	41.0±19.0%	2 (33.3%)	-
Type I deficiency	32 (86.5%)	24 (66.7%)	56 (76.7%)	27 (48.2%)	52.2±9.7%	11 (19.6%)	3 (5.4%)
Type II deficiency	5 (13.5%)	9 (25.7%)	14 (19.2%)	6 (42.8%)	50.2±15.4%	6 (42.8%)	3 (21.4%)
Type II HBS deficiency	1 (2.8%)	7 (19.4%)	8 (11.0%)	2 (25%)	45±19.4%	1 (12.5%)	2 (25%)*

HBS: heparin binding site; CSVT: cerebral sinovenous thrombosis. Unusual thrombosis: renal veins, CSVT, deep veins of upper extremities; *Both patients carried the p.Leu131Phe in homozygosis.
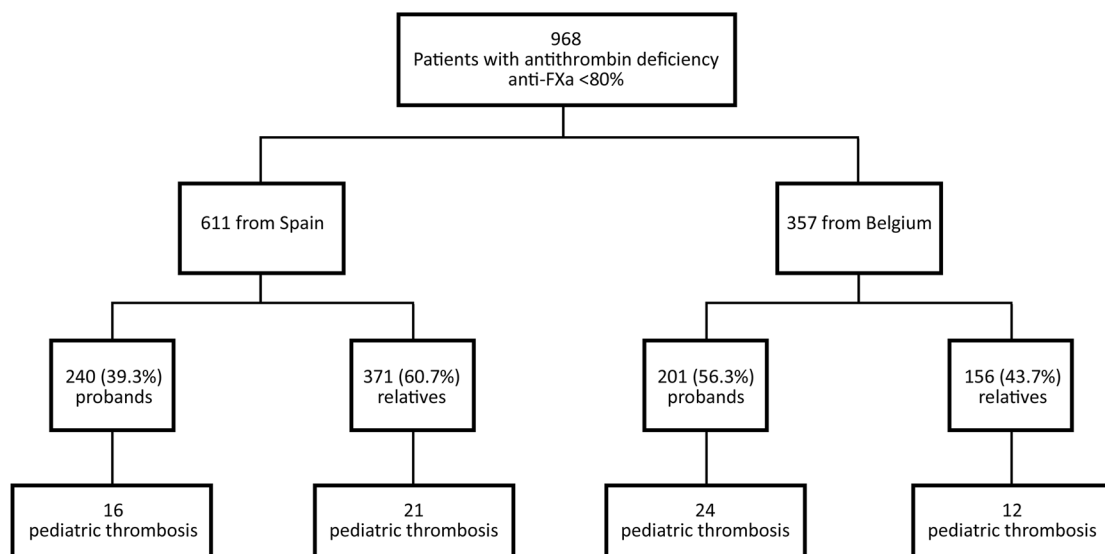

Figure 1. Flow chart of children selected from the entire population. A total of 73 pediatric patients: 40 probands and 33 relatives.

sis-free survival between males and females are also illustrated by Kaplan-Meier survival curves (Figure 3).

Analysis by age revealed two periods with higher prevalence of thrombosis: adolescence (n=49, 67.1%) and the neonatal period (n=15, 20.5%) (Figure 2). In adolescents, clinical presentation was similar to adults: deep vein thrombosis of lower limbs and/or pulmonary embolism (n=47). They also shared the same predisposing factors as adults: pregnancy/puerperium, oral contraceptive use, trauma, immobilization or surgery (n=22) (*Online Supplementary Table S1*).

Remarkably, in neonates, thrombosis often occurred at unusual sites (11 of 15, 73.3%) (Table 1) such as upper extremities, renal veins and cerebral veins. Four patients suffered from arterial thrombosis, with associated venous thrombosis in two of them. In seven neonates, the thrombotic events were idiopathic while in the other eight, possible provoking factors were identified: complicated delivery (forceps or vacuum extraction), infection/sepsis, trauma, surgery or fetal distress (Figure 2 and *Online Supplementary Table S1*). Only one thrombotic event was associated with the presence of a central venous catheter.

The prevalence of cerebral sinovenous thrombosis (CSVT) was very high in our cohort (n=13; 17.8%), especially at a young age (8 neonates and 4 children <6 years) (Figure 2 and *Online Supplementary Table S1*). It is noteworthy that in three cases CSVT occurred after assisted delivery (emergency caesarian section, forceps or vacuum extraction). It is interesting to note the extreme severity of the events. Six children (8.2%) died as a consequence of a thrombotic episode. If we only consider neonatal thrombosis, fatality rate rises to 20% (3 of 15) (*Online Supplementary Table S1*). Interestingly, two of the deceased neonates were unrelated homozygous carriers of the p.Leu131Phe variant, responsible for Antithrombin Budapest III, a type II heparin binding site deficiency.[14] Moreover, morbidity after pediatric thrombosis was severe. One child needed to have an arm amputated,

another developed serious psychomotor retardation, and one had permanent tetraplegia.

From a molecular/biochemical point of view, the symptomatic children in our study predominantly showed type I antithrombin deficiency (76.7%). This results in a significantly higher thrombotic risk associated with type I deficiency compared to type II, with an odds ratio of 2.3 (95%CI: 1.26-4.18; *P*=0.007). Only 14 patients carried a type II deficiency: six were type II RS or PE and eight type II HBS deficiency (*Online Supplementary Table S1*). In patients with type I deficiency, around half of the thrombotic events were unprovoked while this was much higher (75%) among patients with type II HBS deficiency. In most patients (6 of 8) with type II HBS deficiency the p.Leu131Phe variation was detected; four were homozygous and the two heterozygous cases were also carriers of the Factor V Leiden mutation (one heterozygous and one homozygous) (*Online Supplementary Table S1*). Considering the whole cohort of subjects with antithrombin deficiency, only eight out of 223 subjects with type II HBS deficiency (3.6%) suffered from thrombosis during childhood and, as indicated before, most of them carried additional genetic risk factors or had the *SERPINC1* mutation in homozygous state. The prevalence of pediatric thrombosis in the whole cohort of individuals with type I deficiency was higher: 56 out of 604 (9.3%).

In two patients, the molecular mechanism responsible for the antithrombin deficiency was not found. These patients showed low anti-FXa activity on several independent blood samples and had first degree family members with the same low antithrombin values. One patient had a congenital disorder of N-glycosylation as the underlying cause of the deficiency (*Online Supplementary Table S1*).[19]

The p.Leu131Phe mutation was the most prevalent mutation in our pediatric cohort with six carriers belonging to five families. Four unrelated patients carried the c.1154-14G>A mutation affecting splicing and four
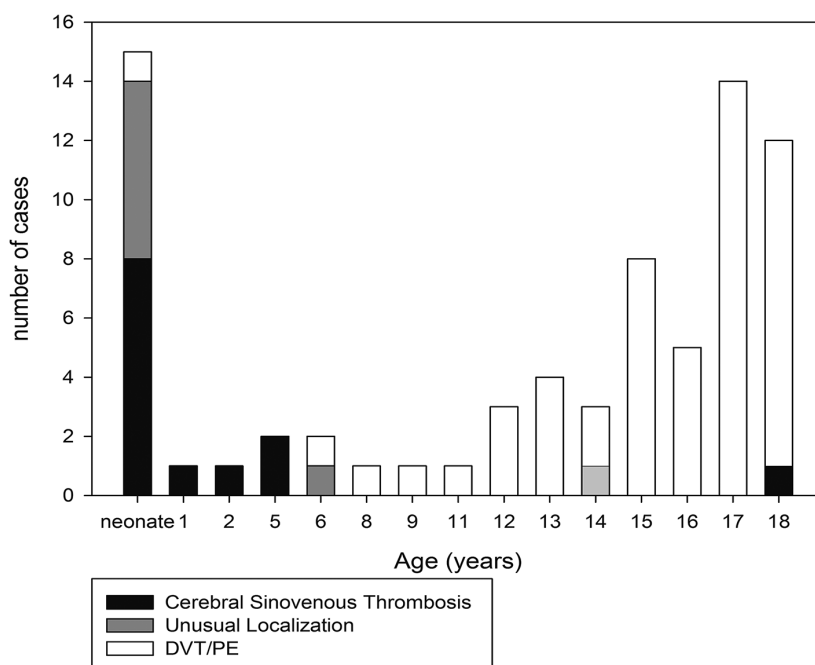

Figure 2. Distribution of thrombotic events among children with antithrombin deficiency according to age. Localization of the thrombosis is also represented: deep vein thrombosis (DVT) of the lower limbs and/or pulmonary embolism (PE) (white), cerebral sinovenous thrombosis (black), or unusual localizations (gray).

patients from two unrelated families presented p.Arg161* mutation. Finally, we identified two families where more than one member developed pediatric thrombosis: three carriers of p.Pro112Ser from the same family developed thrombosis during childhood, but this is a large family with 14 affected members; and two twins carrying p.Ser223Pro developed VTE at 12 and 15 years old. Genetic variants associated with the presence of unusual disulphide-linked dimers in plasma were identified in 14 children: p.Gly456Arg, p.Pro112Ser, p.Pro112Leu, c.1154-14G>A, p.Ser114Asn and p.Ser381Pro. The remaining mutations were predominantly distinct missense or nonsense mutations responsible for type I deficiency (*Online Supplementary Table S1*).

Discussion

The low prevalence of severe thrombophilic disorders like deficiencies of the natural anticoagulants antithrombin, protein C and protein S renders it difficult to estimate the thrombotic risk in patients affected by these conditions. This limitation is even more prominent when considering pediatric thrombosis. In particular, for antithrombin deficiency very few data are available about the occurrence of thrombosis in the first two decades and most information results from case reports or small case series,[13-15] as well as from reports on thrombophilia in large cohorts of pediatric patients.[16,20]

Our results, obtained from the largest series of pediatric antithrombin deficient patients world-wide, emphasize the severity of this condition and suggest that more strict recommendations on the management of families with antithrombin deficiency should be considered.

The incidence of pediatric thrombosis among our antithrombin deficient patient cohort was as high as 7.5%, 4.32 cases/1,000-patient years, or 300-fold higher than described in the general population (0.0014%/year).[4]

We observed more thrombotic complications in males than females (male-to-female ratio 1.2:1), consistent with previous studies in children.[5,16,20-22] Thrombosis in antithrombin deficient children also seems to be age-dependent. In accordance with other studies,[23] we observed a fairly consistent pattern with an initial peak incidence of thrombosis during the neonatal period and a second increment occurring in adolescence. During adolescence, the localization of the thrombosis and the triggering risk factors were similar to those seen in adults, notably estrogen-related conditions (oral contraceptives, pregnancy and puerperium). The reason for the high incidence of thrombosis in neonatal period could be attributed to the labile hemostatic system in newborns with reduced levels of many coagulation factors and inhibitors, including antithrombin. Antithrombin levels are known to be 60% reduced at birth and to reach adult values around three months of age.[24,25] We speculate that the physiological antithrombin deficiency at birth is exacerbated by the addition of a congenital defect of this protein, making the neonate more sensitive to any other prothrombotic triggering factor like acidosis, hypoxia, thermal changes, release of tissue factor, and a frequent exposure to trauma and manipulation. Indeed, in half of the neonates with thrombosis, one of these conditions was present. It is worth mentioning that only one of the initial thrombosis in the neonates from our cohort was catheter-related, while this is the main overall cause of thrombosis in newborns.[26] We note a strikingly high prevalence (17.8%) of cerebral sinovenous thrombosis in our pediatric cohort. This finding is not consistent with prior reports of single cases or small case series.[27,28] It is plausible that the development of the skull (with fontanels) makes the newborn vulnerable to cerebral thrombosis during this period, particularly if associated with thrombophilia and/or localized trauma.[29] Three of the events in our study occurred after assisted delivery, a procedure known to be associated with 60% of the cases of CSVT.[30]

Of interest is the severe outcome and mortality in our pediatric antithrombin deficient patients. Directly attributable mortality was 4-fold higher compared to children with thrombosis from the Canadian Childhood Thrombophilia registry (8.2% *vs.* 2.2%, respectively),[31] including different types of thrombophilia. Other small
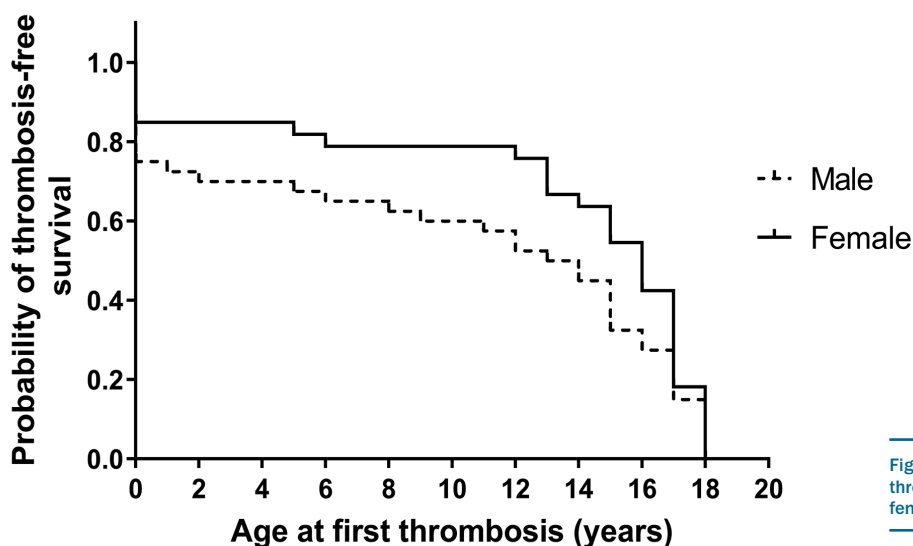

Figure 3. Kaplan-Meier survival curves of thrombosis-free survival between male and female patients with pediatric thrombosis.

studies, restricted to cases with antithrombin deficiency, showed also lower mortality rates.[32,33] Two of the six deceased patients in our cohort were carrying the p.Leu131Phe variant, Antithrombin Budapest III, in homozygous state. The occurrence of pediatric thrombosis associated with this variant present in homozygosity has been described earlier,[14,15,34] but, as far as we know, never with fatal outcome.

The frequency of thrombosis in patients with type I *vs.* type II deficiency was significantly higher with an OR of 2.3 (95%CI: 1.26-4.18; *P*=0.007). Interestingly, the prevalence of type II HBS deficiency was low in our cohort with the majority of patients carrying this specific subtype in homozygous state or having an additional thrombophilic defect. This finding supports the fact that isolated heparin binding site deficiency in heterozygous state is less thrombogenic in children than type I or the other type II deficiencies. There was a remarkable number of patients carrying genetic variants associated with formation of disulphide-linked dimers (14 of 73: 19.2%). The presence of these dimeric forms indicates that the causative mutation has a major impact on the correct folding of antithrombin.[35] These results reinforce the hypothesis that mutations with conformational consequences have severe clinical implications and might also increase the risk of pediatric thrombosis.[36]

It is still a matter of debate whether it is useful to test for thrombophilia in children with a first venous thrombotic event or in asymptomatic children from families with thrombophilia.[37,38] The identification of an inherited thrombophilic defect does not alter the acute antithrombotic management in children,[39] and it is not common practice to administer thromboprophylaxis in children in high-risk situations such as immobilization, surgery, or trauma.[40] However, a recent study suggests that thrombophilia care in children should be individualized.[41] Our study has shown a high incidence of severe thrombotic events in children with antithrombin deficiency, most of them in high-risk situations, supporting the recent recommendation on the screening of thrombophilia in children with positive family history of VTE and/or severe thrombophilia.[41] Thus, we recommend testing for antithrombin deficiency in children of affected families, particularly for those carrying type I deficiency. These carriers might have benefited from preventive strategies like thromboprophylaxis in high-risk situations,[42-46] and from counseling concerning risk factors such as oral contraceptive use. We also propose to test for antithrombin deficiency in pediatric cases with cerebral sinovenous thrombosis or thrombosis occurring at unusual sites. Although this may not change directly the treatment of the thrombotic event, it may provide valuable information for future management of these patients and their family members. In selected cases, antithrombin concentrate could be a valuable treatment option, although this should be validated in clinical trials. Given the high frequency of CSVT in neonates with antithrombin deficiency, we recommend avoiding invasive procedures, like forceps or vacuum extraction, during delivery if one of the parents has antithrombin deficiency.

Our study has certain limitations, some of them due to its retrospective design. For example, in some cases, data on additional thrombophilic factors were lacking. Additionally, although anti-FXa is the method currently recommended for the diagnosis of antithrombin deficiency, there are some specific mutations that can only be detected by anti-FIIa or molecular methods.[47,48] Accordingly, as the screening method used to identify the patients in our cohorts was anti-FXa, our study could have missed some cases of antithrombin deficiency, whose role in pediatric thrombosis has not been evaluated. Similarly, pediatric patients with asymptomatic thrombosis have not been included in our study. Nevertheless, this study represents the largest cohort of pediatric patients with antithrombin deficiency with thrombotic complications reported to date. Our results emphasize the severity of the disorder in the pediatric population and reveal age-dependent differences in thrombotic manifestations and risk factors.

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